

# An evaluation of the effect of exogenous glycinebetaine on the growth and yield of soybean: timing of application, watering regimes and cultivars

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## Abstract

Various soybean cultivars were grown under different watering regimes in the field and greenhouse in south-eastern U.S.A. (1995 and 1996), and in the field in north-eastern Western Australia (1995). Aqueous glycinebetaine was applied at different growth stages onto their foliage with the objective of ameliorating effects of water stress on photosynthesis activity, nitrogen fixation, leaf growth, biomass accumulation and seed yield. There were cultivar differences in response to drought. Trends which suggest that exogenous glycinebetaine could improve photosynthesis activity, nitrogen fixation and leaf area development, were established. The observed seed yield increase of both well-watered and drought-stressed plants was associated with greater number of seeds following the application of 3 kg ha<sup>-1</sup> glycinebetaine. The results indicate that foliar-applied glycinebetaine possesses anti-transpirant properties and has the potential to improve drought tolerance and reduce the amount of water used for irrigation, without any significant decrease in economic yield. There is evidence that soybean could be classified as a low-accumulator of glycinebetaine. © 1997 Elsevier Science B.V.

**Keywords:** Acetylene reduction; Anti-transpirant; Drought; Leaf diffusive resistance; Nitrogen fixation; Photosynthesis; Soybean

## 1. Introduction

Soybean (*Glycine max* L. Merrill) is the most important grain legume and oilseed crop, providing approximately 60% of the world's supply of vegetable protein and 30% of oil (Fehr, 1989). Although worldwide productivity has increased significantly in the last two decades, moisture stress remains the

most important limitation for yield. Reduction in seed yield is associated with the multiplicative effects of water deficit, such as decreases in leaf area development (Muchow et al., 1986), net photosynthesis rate (Bunce, 1988; Frederick et al., 1989), symbiotic dinitrogen fixation (Weisz et al., 1985) and biomass accumulation (Sinclair et al., 1987). The extent to which yield is affected depends on the severity, timing and duration of the water deficit (Kpoghomou et al., 1990), and cultivar (Bunce, 1988; Frederick et al., 1989). Irrigation is widely adopted

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in environments where extended periods of drought are common, such as in subtropical areas of Australia (Lawn et al., 1986), and where mid-summer drought occurs frequently, as in the south and mid-west of the United States of America (Bowers et al., 1989).

One of the adaptation mechanisms suggested for reducing vulnerability to drought is the lowering of osmotic potential (Ludlow and Muchow, 1990). Osmotic adjustment in some plant species is achieved by the accumulation of solutes such as proline and glycinebetaine (Borowitzka, 1981; Wyn Jones and Storey, 1981). Glycinebetaine (N,N',N''-trimethylglycine) is a quaternary ammonium compound accumulated by many species of the Amaranthaceae, Asteraceae, Chenopodiaceae, Convolvulaceae, Gramineae, Malvaceae, Poaceae, and Portulacaceae families (Wyn Jones and Storey, 1981; Weretilnyk et al., 1989). There is good evidence to suggest that it acts as a non-toxic cytoplasmic osmolyte and plays a central role in adaptation to stress (Dawson et al., 1969; Yancey et al., 1982; Wyn Jones, 1984).

Even though glycinebetaine-positive lines of some species such as maize (Brunk et al., 1989; Rhodes et al., 1989), wheat (Naidu et al., 1990), and sorghum (Grote et al., 1994) have been identified, breeding for glycinebetaine accumulation has been hampered by the inability to isolate the dominant allele determining stress-induced glycinebetaine accumulation (Rhodes et al., 1989). Glycinebetaine is metabolically inert and readily translocated from its site of synthesis in the leaves to the other parts of the plant (Ladyman et al., 1980; Hanson and Wyse, 1982). However, it is rapidly degraded extracellularly by soil microbes (Kortstee, 1970; Wyn Jones et al., 1973).

Although traces of glycinebetaine have been detected in grain legumes such as common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (Takhtajan, 1980), no report is available that soybean accumulates glycinebetaine. Cultivar differences notwithstanding, soybean has a limited capacity to avoid drought (Sinclair et al., 1987) and breeding for this trait is laborious and expensive (Blum, 1987). The corollary is to use rapid and relatively simple methodologies that could bring about a positive shift in productivity of soybean under drought.

Results from recent field and greenhouse experi-

ments have shown exogenous glycinebetaine to improve drought tolerance of tobacco (*Nicotiana tabacum* L.) (Agboma et al., 1996b) and lupin (*Lupinus angustifolius* L.) (Agboma et al., 1996c); furthermore, it increased leaf area index of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Mäkelä et al., 1996a). There are indications that glycinebetaine could compensate grain yield for a reduction in the amount of water needed for irrigation in maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) (Agboma et al., 1996a). The studies also indicated that rate and timing of glycinebetaine application affect the outcome, and that crops respond differentially to soil water status.

The current study is part of research into the use of exogenous glycinebetaine to ameliorate the effects of water deficit on crops grown in water-limiting conditions. It is designed to complement the reliability of field experiments with the precision of controlled environments, especially as plants under water stress respond differently in the field and in controlled environments (Jordan and Ritchie, 1971; Jarvis and McNaughton, 1986), partially because root volumes and available soil water per plant are usually greater in the field (Hall, 1990). Furthermore, the study seeks to address the question of whether soybean accumulates glycinebetaine in response to water stress.

## 2. Material and methods

### 2.1. Greenhouse experiments

#### 2.1.1. Leaf photosynthesis activity

Seeds of soybean cv. Biloxi inoculated with *Rhizobium japonicum* (Nitragen Co., Milwaukee, WI), were sown on June 11, 1995 in pots, 12 cm diameter and 14 cm high, containing garden-mix soil, in the greenhouse at the Department of Agronomy, University of Florida, Gainesville (29°39'N, 82°20'W), USA. After germination, the plants were thinned to one per pot and well watered daily. Mean maximum day and night temperatures were 28 and 21°C, respectively. Illumination was provided by natural light and relative humidity fluctuated between 42 and 51%. At 56 days after sowing (DAS) (development stage R1), sixteen plants of uniform height and leaf

number were selected. Aqueous 0.1 M glycinebetaine (as the inner hydroxide salt, pH 7.8, purity 97%, Cultor Ltd Finnsugar Bioproduct, Finland), containing 0.1% of Tween 20 (Fluka Chemie, Buchs, Switzerland) as surfactant, was applied to run-off onto the foliage of 8 of the plants, using a hand-held manual atomizer. The other 8 plants received water and Tween 20. Two days later, 2 watering regimes were introduced: 4 plants in each glycinebetaine treatment remained in well-watered conditions, while the others were stressed by replacing 80% of water lost through evapotranspiration daily during the first 2 days, and then dried further by withholding rewatering for the next 2 days.

Measurements of the rate of leaf photosynthesis were conducted at 58, 59, 60 and 61 DAS using an LI-6200 Portable Photosynthesis System (Li-Cor, Lincoln, NE). On each occasion, the plants were moved outside the greenhouse into sunlight at about midday and left for 2 h to stabilize. When the photosynthetic photon flux density was more than  $1500 \mu\text{mol s}^{-1} \text{m}^{-2}$ , net photosynthesis of a sun-attenuated leaflet of the uppermost fully expanded leaf was recorded. The detachment of the observed leaflets for area measurement made it necessary to also take readings from the leaflets of the penultimate fully expanded leaves.

### 2.1.2. Nitrogen fixation

Single soybean seeds (cv. Biloxi) were sown on July 18, 1995 in the greenhouse at the Department of Agronomy, University of Florida, Gainesville, USA, in 20 polyvinyl chloride (PVC) pots, containing a garden-mix soil that had been inoculated with *R. japonicum*. The pots were 10 cm in diameter and 20 cm high. Covers made of 1-cm-thick clear acrylic, were constructed in two halves with central notches to fit around the plant stems, and securely fastened to the tops of each pot. Caulking putty was used to seal around the plant stems to the pot covers. Evaporation and gas exchange were thus negligible. Growth conditions were as in the leaf photosynthesis experiment above.

The plants were well-watered daily until 18 DAS (stage V6) and nodulated well. Approximately 160 ml of aqueous glycinebetaine (0.10 M), containing 0.1% of Tween 20 as surfactant, was applied to run-off onto the foliage of 10 plants using a hand-held

manual atomizer. The remaining 10 plants were sprayed with water and surfactant. Two days later, 2 watering regimes were imposed on each set of plants: 5 plants remained well watered, while the other 5 were subjected to a phase of soil drying, accomplished by replacing 80% of water lost through transpiration.

At 23 and 24 DAS, estimates of nitrogen fixation activity in the root nodules were made by acetylene reduction using a flow-through system in the pots (Sall and Sinclair, 1991). A gas mixture of 1:9 v/v acetylene:air was pumped at  $1 \text{ l min}^{-1}$  into each pot through an entrance port at the bottom of the pot. The gas mixture exited the pot through a port in the lid, which also served as the site for collecting gas samples. A gas distribution system was constructed so that the 20 pots could be monitored simultaneously.

The gas mixture flowed through the pots for a 10 min equilibration period before the gas sample was collected from the exit port. Following sampling, air was pumped through the pots for at least 45 min to flush the pots of acetylene and ethylene. The ethylene peak in the gas sample was integrated using a gas chromatograph (Hewlett-Packard Model 5710A), with a flame ionization detector. Nitrogen-fixation was estimated by converting the area of each ethylene peak to parts per million.

### 2.1.3. Soil drying cycle

Single soybean seeds (cv. Biloxi) were sown on December 26, 1995 (in same greenhouse and under same conditions as for previous experiment), in PVC pots, whose tops were covered by acrylic to minimize soil evaporation. The pots were watered to field capacity every other day. At 37 DAS, twenty uniform plants were selected. Leaves of 8 plants were sprayed until drip-off (ca. 20 ml) with water containing 0.1 M glycinebetaine and 0.1% Tween 20. Eight other plants were sprayed with water and Tween 20. The remaining 4 plants were left untreated and subsequently used as the well-watered controls. All pots were watered to saturation point at 41 DAS, and allowed to drain overnight. The well-watered weights of the individual pots were then obtained. During the subsequent 12 days, the soil in all pots, except for the control, was allowed to dry through transpiration.

Each afternoon during the drying cycle, the pots were weighed and the difference between successive days was calculated as the daily transpiration rate. The pot weight on each day was used to calculate the fraction of transpirable water in each pot (Sinclair and Ludlow, 1986). The total transpirable soil water for each pot was calculated as the difference between the 'well-watered' weight and the weight when transpiration had decreased to 10% of the control plants. Small amounts of water were added to the pots each day to reestablish the moisture status in all of the drying pots to roughly equal fractions of transpirable soil water. Nitrogen fixation activity of each plant was measured each afternoon by the continuous-flow acetylene reduction method as previously described.

## 2.2. Field experiment 1

### 2.2.1. Site and crop management

Two soybean cultivars, Cook and Biloxi, were sown on April 12, 1995 in 0.91 m rows with seeds 2 cm apart in a USDA classification Arredondo fine sand soil (that had previously supported several soybean crops), at the Irrigation Research Park, University of Florida, Gainesville, FL, USA. Prior to sowing, the soil was fertilized with 180, 240 and 900 kg ha<sup>-1</sup> N-P-K, respectively, and micronutrients. During establishment, the plants were irrigated to field capacity every 3 to 5 days (depending on rainfall), using overhead sprinklers that applied 24 mm of water h<sup>-1</sup>. Plots measuring 6.8 × 6.8 m, and containing 6 rows of plants, were completely randomized in 4 replicates, with 2 irrigation treatments as the main plots. Two glycinebetaine rates (3 and 6 kg ha<sup>-1</sup>) and a control were the subplots in the design.

At 32 DAS, when plants were at development stage V11 (Fehr and Caviness, 1977), 3 and 6 kg of glycinebetaine were dissolved and applied in 410 l of water ha<sup>-1</sup> (7 and 15 mM solutions, respectively), onto the foliage of the crop using a back-mounted pressurized sprayer (Weed Systems, Gainesville, FL). 1 ml of Tween 20 was added as a surfactant to a liter of the solution prior to spraying. Control plants were sprayed with water and Tween 20. Two days later, water treatment was imposed. One of the main plots was watered regularly to recharge the soil profile, which had an inherently low water retention capability.

Table 1

Supplementary watering (mm) received during trial period by the two water treatments at Gainesville, Florida, in 1995. Amount was dependent on rainfall (mm)

Date	Water treatments		Rainfall
	Limited watered	Well-watered	
May 4	25	25	—
9	25	25	—
12	—	—	38.9
13	—	—	20.1
16	—	25	—
19	—	25	—
20	—	—	3.8
21	10	—	—
24	10	25	—
27	—	25	—
28	10	—	—
30	—	25	—
June 1	10	—	—
2	—	25	0.5
3	—	—	11.7
4	—	—	19.6
5	—	—	44.5
6	—	—	3.8
9	—	25	—
12	—	25	—
13	—	—	2.8
14	10	—	—
16	—	25	—
18	10	—	—
19	—	25	—
20	—	—	11.7
22	—	25	—
23	—	—	58.9
25	—	—	2.0
26	—	—	39.1
27	—	—	18.5
28	—	—	3.6

ity. Watering was withheld in the stressed treatment, until partial midday stomatal closure (indicated by leaf droop) was observed, and then rewatered with 10 mm, so that the plants would again quickly be subjected to stress. The frequency of watering was dependent on rainfall (Table 1) and was such that the stressed plants were watered 6 times with 10 mm. In contrast, the well-watered control received eleven waterings each of 25 mm.

### 2.2.2. Measurements

At 29, 43 and 56 DAS, phytomass of plants from adjacent 50-cm lengths of each 4 inner rows (1.82

m<sup>2</sup>) in each plot were harvested and oven-dried to constant weight. At each harvest, leaf area of 4 other plants was measured with a leaf area meter (Model LI-3100, Li-Cor, Lincoln, NE). The leaves were dried to constant weight and samples were analyzed for glycinebetaine concentration at Cultor Ltd Technology Center, Kantvik, Finland using high performance liquid chromatography (Rajakylä and Palo-  
poski, 1983), with the exchange column in Ca<sup>2+</sup> form.

On eight occasions between 29 and 76 DAS, incident solar radiation just above and below the crop canopy was measured between 1300 and 1400 h, using a 1 m line sensor (Model LI-191S, Li-Cor, Lincoln, NE). The interception percentage derived from the difference between the two readings at 29 and 43 DAS (first and second phytomass harvest dates) were used to calculate total radiation received for the period. Radiation-use efficiency (RUE) in accumulation of above-ground biomass was also calculated for the period as the ratio of the biomass

accumulated to the cumulative daily amount of photosynthetically active radiation (PAR), given on the climatological data (University of Florida, Gainesville, Meteorological Services).

On clear days, abaxial leaf diffusive resistance (inverse of leaf conductance) was measured between 1300 and 1400 h on the middle leaflet of the uppermost fully expanded, sun-attenuated leaf, randomly selected in each plot, using an LI-1600 Steady State Porometer (Li-Cor, Lincoln, NE) whose cuvette was designed to measure a surface at a time. Between 44 and 86 DAS, 10 sets of measurements of randomly selected plants were undertaken.

At 35 and 36 DAS, the acetylene reduction assay (Vessey, 1994) was undertaken at noon to estimate nitrogen fixation activity in root nodules. A metal cylinder (10 cm diameter) was pushed to a depth of 20 cm of the soil to remove two plants with their roots and nodules intact. Soil was carefully shaken off the roots. The shoots were cut off and the roots promptly placed inside a glass jar (vol. 960 ml),

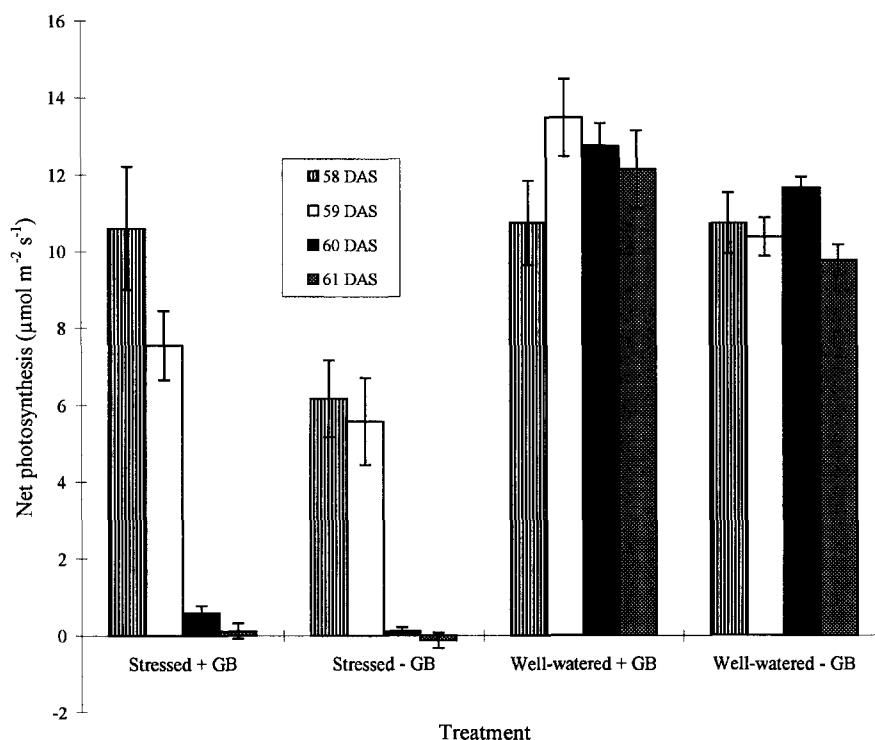


Fig. 1. Effect of foliar-applied 0.1 M glycinebetaine (GB) on leaf photosynthesis of soybean cv. Biloxi. Bars are standard deviations of the populations.

which was then tightly capped. Approximately 120 ml of air was withdrawn from inside the jar using a hypodermic needle through a rubber stopper screwed into the jar cap. Another syringe was used to place 100 ml of 1:9 v/v acetylene:air mixture into the closed jar. After five minutes of incubation, a 10 ml sample of the gas mixture in the jar was withdrawn and injected into a vacuutainer that had been cleared of its air content. This gas withdrawal procedure was repeated after 10 and 15 min of root incubation. The concentration of ethylene (ppm) in each vacuutainer was determined as for the greenhouse assay. Ethylene production was plotted against time and the slope of the regression was obtained to estimate rate of ethylene production.

### 2.3. Field experiment 2

#### 2.3.1. Site and crop management

Soybean (cv. Manark) was inoculated with *R. japonicum* (Nitrogerm, Bio-care Tech. Pty. Ltd.,

Somersby, NSW, Australia) and sown on June 23, 1995 at Kununurra ( $15^{\circ}42'S$ ,  $128^{\circ}50'E$ ), north Western Australia, in a gray-black, silty clay alfisol, pH 6.7. Nitrogen at a rate of  $100\text{ kg ha}^{-1}$  was incorporated into the soil at sowing. Plot size was 5 m of bed, each containing 6 plant rows 0.9 m apart, with circa  $11.5\text{ plants m}^{-2}$ . The experimental design was in 3 randomized complete blocks for each watering regime. One block was optimally irrigated, while the other 2 received either 50 or 75% of the optimum water supply. There were 4 subblocks per block, each subblock containing a replicate of the timing/glycinebetaine treatment, with  $E_1$ ,  $E_3$  and  $E_6$  representing 1, 3 and  $6\text{ kg ha}^{-1}$  (equivalent to 4, 12 and  $24\text{ mM}$  solutions) of glycinebetaine, respectively, applied at full bloom (stage R2) and  $F_1$ ,  $F_3$  and  $F_6$  representing 1, 3 and  $6\text{ kg ha}^{-1}$  glycinebetaine, respectively, applied at pod set (stage R3).

The plots were irrigated to field capacity at fortnightly intervals between 2 and 34 DAS. The watering regimes were introduced at 44 DAS, when the

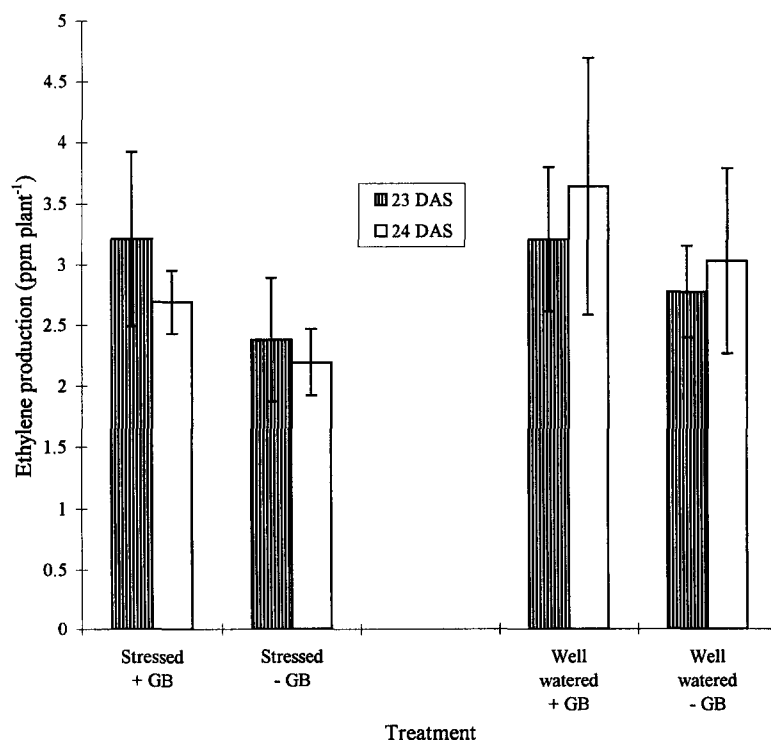


Fig. 2. Effect of foliar-applied 0.1 M glycinebetaine on ethylene production (nitrogen fixation) of soybean cv. Biloxi. Bars are standard deviations of the populations.

Table 2

Daily transpiration ( $\text{ml d}^{-1}$ ) of glycinebetaine-treated and untreated soybean (cv. Biloxi) plants under a soil drying cycle, relative to well-watered, non-glycinebetaine treated plants at Gainesville, Florida, in 1996

Day	0.1 M Glycinebetaine (A)	Untreated (B)	Ratio A:B
1	0.81	0.94	0.86
2	0.81	0.98	0.83
3	0.89	1.05	0.85
4	0.88	1.03	0.85
5	0.79	0.92	0.86
6	0.70	0.83	0.84
7	0.69	0.83	0.83
8	0.44	0.51	0.86
9	0.33	0.39	0.85
10	0.22	0.26	0.85
11	0.17	0.22	0.77

plants were at stage R1. Water supply channels along the sides of, and in the blocks, were filled to capacity, enabling the buffer furrows alongside the experimental rows to fill within 10 minutes after the check bank was breached. Allowing for the decline in infiltration rate with time, the 50, 75 and 100% irrigation regimes drained in 2.5, 4 and 6 h, respectively.

At 46 DAS, glycinebetaine treatments were applied to  $E_1$ ,  $E_3$  and  $E_6$  using a pressurized sprayer (Spraying Systems Co., Melbourne, Australia), designed to deliver the pre-determined volume of  $250 \text{ l ha}^{-1}$  of solution at a nozzle discharge rate of  $5.9 \text{ ml s}^{-1}$ . The surfactant Plus 50 (Ciba Geigy, Basle, Switzerland) was added to all sprays at  $2 \text{ ml l}^{-1}$ . The control received water and surfactant. A week later,  $F_1$ ,  $F_3$  and  $F_6$  received the same glycinebetaine treatment. The water treatment was terminated on September 3 (72 DAS).

### 2.3.2. Measurements

The plots were harvested when the plants had ceased growth and pods were filled at 80 DAS. Plants along a 1 m length of the 2 inner rows in the plots were cut at ground level and weighed. Pods were collected, counted and weighed. The stripped plants and pods were dried to constant weight at  $80^\circ\text{C}$ . The pods were threshed and the seeds were counted and weighed. They were sorted into 2 sizes: large (could not pass through a 5.66 mm wire mesh),

and small. The sorted seeds were also counted and weighed.

### 3. Statistical analysis

Differences in water and glycinebetaine treatments for leaf area expansion, leaf nitrogen content, biomass accumulation, leaf resistance, canopy characteristics and field nitrogen fixation for each cultivar in the Florida experiments, were subjected to analysis of variance (MSTAT Development Team, 1989). The means were separated and ranked using

Table 3

Effects of water and glycinebetaine treatments on leaf area ( $\text{cm}^2 \text{ plant}^{-1}$ ), leaf and phytomass dry weight ( $\text{g plant}^{-1}$ ) of two field-grown soybean cultivars at Gainesville, Florida, in 1995. Within columns, numbers with the same letters are not significantly different at  $P = 0.05$  (Student–Newman–Keul's test)

Treatment	Leaf area		Leaf dry weight		Phytomass dry weight	
	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi
29 DAS						
S B <sub>0</sub>	648 a	576 c	2.31 a	1.76 cd	3.45 a	2.42 b
S B <sub>3</sub>	524 a	644 c	1.76 a	1.93 bc	3.38 a	2.64 ab
S B <sub>6</sub>	592 a	599 c	2.11 a	1.81 bcd	3.08 a	2.46 b
W B <sub>0</sub>	788 a	809 a	2.54 a	2.22 a	3.94 a	3.22 ab
W B <sub>3</sub>	752 a	817 a	2.61 a	2.55 a	3.68 a	3.18 ab
W B <sub>6</sub>	748 a	717 b	2.43 a	2.00 ab	3.71 a	3.75 a
Source	$P > F$					
Water	—	0.009	—	0.004	—	0.032
43 DAS						
Treatment	Leaf area		Leaf DW		Above-ground DW	
	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi
S B <sub>0</sub>	812 a	926 b	2.78 ab	2.61 b	6.30 a	4.64 b
S B <sub>3</sub>	942 a	991 b	3.16 ab	2.82 ab	5.10 a	4.12 b
S B <sub>6</sub>	721 a	895 b	2.56 b	2.64 b	5.39 a	4.33 b
W B <sub>0</sub>	886 a	1533 a	3.37 ab	3.78 ab	7.66 a	6.91 a
W B <sub>3</sub>	1099 a	1502 a	3.54 ab	4.08 a	7.43 a	6.16 a
W B <sub>6</sub>	1431 a	1610 a	4.42 a	3.87 ab	9.05 a	6.16 a
Source	$P > F$					
Water	—	0.001	0.033	0.009	—	0.014

B<sub>0</sub>, B<sub>3</sub> and B<sub>6</sub>: control, 3 and 6  $\text{kg ha}^{-1}$  glycinebetaine, respectively.

S, water-stressed.

W, well-watered.

Table 4

Effect of water regime on amount of photosynthetically active radiation (PAR) intercepted and radiation-use efficiency (RUE) of two soybean cultivars for the period between 29 and 43 DAS, at Gainesville, Florida, in 1995. Within columns, numbers followed by the same letters are not significantly different at  $P = 0.05$  (Student–Newman–Keul's test). Significance corresponds to radiation interception % based on canopy closure

Plant status	PAR intercepted (MJ m <sup>-2</sup> )		RUE (g MJ <sup>-1</sup> )	
	cv. Cook	cv. Biloxi	cv. Cook	cv. Biloxi
Water-stressed	121.7 b	136.0 b	0.53	0.35
Well-watered	159.0 a	154.6 a	0.57	0.55

Student–Newman–Keul's (SNK) test,  $P = 0.05$ . For the Australian experiments, significance of differences in biomass production and seed yield due to rate and timing of glycinebetaine application for the 3 irrigation regimes, were also established with anal-

ysis of variance. Means were separated and ranked using the Least Significant Difference (LSD) test,  $P = 0.05$ . Due to the similarity of the results obtained, data for the 75 and 100% irrigation levels were pooled and reanalyzed.

## 4. Results

### 4.1. Greenhouse experiments

Although the number of plants from which data were obtained was small, clear trends of the effects of the treatments were established. Leaf photosynthesis activity of cv. Biloxi was significantly reduced after the imposition of water deficit (Fig. 1). Differences in net photosynthesis and nitrogen fixation (Figs. 1 and 2), following glycinebetaine application were, however, not significant ( $P = 0.05$ ). The daily

Table 5

Effect of water and glycinebetaine treatments on abaxial leaf diffusive resistance (s cm<sup>-1</sup>) of the uppermost fully expanded leaves of two field-grown soybean cultivars between 1300 and 1400 h at Gainesville, Florida, in 1995. Readings were taken irregularly between 44 and 87 DAS. Within columns, numbers followed by the same letters are not significantly different at  $P = 0.05$  (Student–Newman–Keul's test)

Treatment	44 DAS		45 DAS		46 DAS		49 DAS		61 DAS	
	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi
S B <sub>0</sub>	6.96 a	6.03 a	7.29 a	7.48 a	2.03 a	1.75 ab	5.77 a	6.09 a	5.20 a	7.32 a
S B <sub>3</sub>	8.27 a	7.42 a	9.25 a	9.84 a	2.62 a	2.10 a	5.66 a	7.85 a	6.98 a	4.46 ab
S B <sub>6</sub>	8.82 a	5.85 a	7.96 a	7.32 a	2.19 a	1.68 ab	4.56 a	7.46 a	5.91 a	4.66 ab
W B <sub>0</sub>	2.26 b	3.30 ab	1.16 b	1.52 b	1.11 b	1.24 ab	0.78 b	0.82 b	0.70 b	0.67 b
W B <sub>3</sub>	1.20 b	0.87 b	1.32 b	1.07 b	0.94 b	1.04 b	0.95 b	0.89 b	0.90 b	0.96 b
W B <sub>6</sub>	1.09 b	1.09 b	1.09 b	1.41 b	0.63 b	1.27 ab	0.89 b	0.68 b	0.81 b	0.89 b
Source	$P > F$									
Water	0.014	0.042	0.002	0.004	0.048	0.018	0.014	0.021	0.004	0.006

Treatment	66 DAS		70 DAS		77 DAS		81 DAS		87 DAS	
	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi	Cook	Biloxi
S B <sub>0</sub>	1.19 a	4.13 a	3.79 a	2.05 bc	0.50 a	0.68 ab	1.79 b	1.59 b	0.84 c	0.66 c
S B <sub>3</sub>	2.42 a	3.43 ab	1.97 ab	4.06 a	0.60 a	0.57 b	2.90 ab	2.45 b	0.84 c	0.81 c
S B <sub>6</sub>	2.97 a	1.84 bc	3.43 a	3.52 ab	0.51 a	0.50 b	1.80 b	2.02 b	1.50 bc	2.59 bc
W B <sub>0</sub>	2.11 a	1.06 c	0.59 b	0.72 c	0.74 a	0.87 a	4.49 ab	5.30 ab	3.98 ab	1.84 bc
W B <sub>3</sub>	1.98 a	1.43 c	0.74 b	0.77 bc	1.05 a	0.49 b	6.33 a	7.34 a	4.74 a	4.49 ab
W B <sub>6</sub>	2.20 a	1.17 c	0.69 b	0.52 c	0.90 a	0.70 ab	5.23 ab	6.86 a	4.87 a	5.75 a
Source	$P > F$									
Water	—	0.018	0.048	0.001	—	0.043	0.046	0.015	0.001	0.002
Glycinebetaine	—	—	—	—	—	—	—	—	—	0.019

B<sub>0</sub>, B<sub>3</sub> and B<sub>6</sub>: control, 3 and 6 kg ha<sup>-1</sup> glycinebetaine, respectively.

S, water-stressed.

W, well-watered.



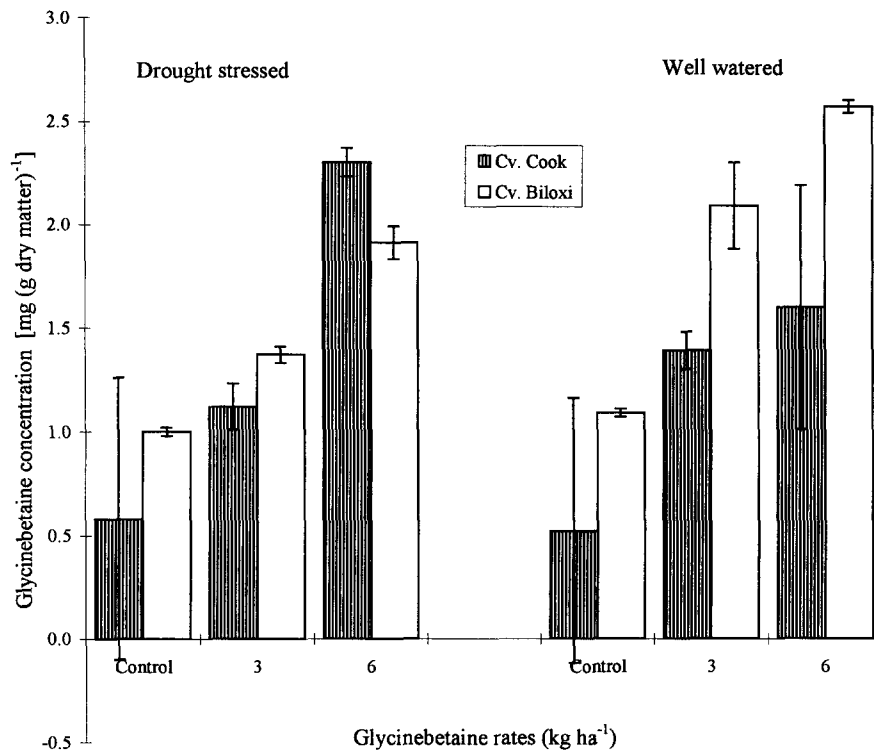


Fig. 3. Leaf glycinebetaine content of soybean after 2 weeks of application. Bars are standard deviations of the populations.

transpiration rates of the glycinebetaine treated plants in the soil drying experiment were relatively lower than those of plants not treated (Table 2). However, the pattern of decreasing transpiration rate and nitrogen fixation (data not shown), when expressed as a fraction of transpirable soil water was equivalent for those plants treated with glycinebetaine and those not treated.

#### 4.2. Field experiment 1

The period of experimentation was characterized by high daytime temperatures (25 to 38°C) and infrequent rainfall. Daytime relative humidity ranged from 30 to 89%. All plots were essentially free of weeds and diseases.

There were varietal differences in response to the limited watering, with respect to leaf area development and above-ground biomass, with cv. Biloxi being more susceptible to stress than cv. Cook (Table 3). Limited watering did not affect the ability of

Table 6

Effects of water and glycinebetaine treatments on regression slopes of ethylene gas production ( $\mu\text{moles plant}^{-1}$ ), against time of root incubation at Gainesville, Florida, in 1995. Gas samples were taken every 5 min for 15 min. Within columns, numbers with the same letters are not significantly different at  $P = 0.05$  (Student–Newman–Keul's test)

Treatment	Regression slope (35 DAS)		Regression slope (36 DAS)	
	Cook	Biloxi	Cook	Biloxi
S B <sub>0</sub>	0.028 a	0.013 a	0.028 a	0.026 c
S B <sub>3</sub>	0.040 a	0.021 a	0.035 a	0.022 c
S B <sub>6</sub>	0.022 a	0.019 a	0.023 a	0.024 c
W B <sub>0</sub>	0.036 a	0.038 a	0.045 a	0.047 a
W B <sub>3</sub>	0.044 a	0.040 a	0.052 a	0.039 b
W B <sub>6</sub>	0.034 a	0.034 a	0.034 a	0.036 b
Source	$P > F$			
Water	—	—	—	0.026

B<sub>0</sub>, B<sub>3</sub> and B<sub>6</sub>: control, 3 and 6 kg ha<sup>-1</sup> glycinebetaine, respectively.

S, water-stressed.

W, well-watered.

cv. Cook to convert PAR into above-ground biomass (Table 4). Although cv. Cook intercepted 11% less PAR than cv. Biloxi, it had a RUE which was one and a half times greater. There were no differences in PAR intercepted and RUE between the cultivars in well-watered conditions. Leaf resistance (inverse of leaf conductance) was significantly elevated by water deficit (Table 5). There were two occasions (65 and 76 DAS) when water deficit had no significant effect on cv. Cook in respect of leaf resistance. The use of glycinebetaine often resulted in higher resistance, especially in the stressed leaves.

Leaf glycinebetaine concentration after 2 weeks of treatment was higher in cv. Biloxi than cv. Cook, except when 6 kg ha<sup>-1</sup> was applied to stressed plants (Fig. 3). The non-treated plants also exhibited relatively high concentrations. After 4 weeks of application, no detectable amount of glycinebetaine was found in the stressed leaves of cv. Biloxi treated

with either 3 or 6 kg ha<sup>-1</sup> glycinebetaine, but a trace amount was present in the leaves of cv. Cook treated with 6 kg ha<sup>-1</sup> (data not shown). An appreciable amount [2.85 mg (g dry matter)<sup>-1</sup>] was, however, detected in well watered plants of cv. Biloxi (data not shown). There were incidences of leaf scorching following the application of 6 kg ha<sup>-1</sup> glycinebetaine, but the effects faded as water treatment progressed.

The regression slope for rate of ethylene production (nitrogen fixation) when the soil had dried for an additional day, portrayed cv. Biloxi as being more sensitive to water deficit than cv. Cook (Table 6). Due to the early sowing date, most plants were barren and thus seed yield data could not be recorded.

#### 4.3. Field experiment 2

The trial period was characterized by high day temperatures (between 28 and 42°C) and lack of

Table 7

Effects of rate and time of glycinebetaine application on pod biomass (g plant<sup>-1</sup>) and pod setting (plant<sup>-1</sup>) of soybean cv. Manark under three irrigation levels at Kununurra, Western Australia, in 1995

Code	Treatment	Fresh weight of pods			Dry weight of pods			No. of pods		
		Irrigation level			Irrigation level			Irrigation level		
		50%	75%	100%	50%	75%	100%	50%	75%	100%
A	E <sub>1</sub>	34.2	42.6	65.8	14.6	16.7	22.1	32	31	43
B	E <sub>3</sub>	44.0	57.8	72.3	19.9	21.1	24.6	41	44	44
C	E <sub>6</sub>	37.9	46.7	57.6	18.2	18.1	20.6	38	35	40
D	F <sub>1</sub>	40.5	47.6	56.8	18.1	16.5	17.3	38	32	41
E	F <sub>3</sub>	39.1	54.3	60.5	16.8	20.5	21.7	35	41	37
F	F <sub>6</sub>	36.4	50.1	56.1	14.6	19.3	19.8	30	39	40
G	Control	46.9	45.2	56.4	20.3	20.1	22.3	40	39	42
	<i>F</i> (prob.)	0.005	0.063	0.317	0.014	0.451	0.446	0.035	0.161	0.890
	LSD ( <i>P</i> = 0.05)	6.08	—	—	3.53	—	—	6.97	—	—
	A–C	38.7	49.0	65.2	17.6	18.6	22.4	37	37	42
	D–F	38.7	50.7	57.8	16.5	18.8	19.6	34	37	39
	<i>F</i> (prob.)	0.751	0.684	0.089	0.250	0.967	0.100	0.103	0.970	0.413
	A × D	37.4	45.1	61.3	16.4	16.6	19.7	35	32	42
	B × E	42.0	56.1	66.4	18.4	20.8	23.2	38	43	41
	C × F	37.2	48.4	56.9	16.4	18.7	20.2	34	37	40
	<i>F</i> (prob.)	0.190	0.026	0.263	0.364	0.120	0.216	0.289	0.071	0.936
	LSD ( <i>P</i> = 0.05)	—	8.4	—	—	—	—	—	—	—

E<sub>1</sub>, E<sub>3</sub> and E<sub>6</sub>: application of 1, 3 and 6 kg ha<sup>-1</sup> glycinebetaine at full bloom (stage R2).

F<sub>1</sub>, F<sub>3</sub> and F<sub>6</sub>: application of 1, 3 and 6 kg ha<sup>-1</sup> glycinebetaine at pod initiation (stage R3).

Table 8

Effects of rate and time of glycinebetaine application on grain yield ( $\text{g plant}^{-1}$ ) of soybean cv. Manark under three irrigation levels at Kununurra, Western Australia, in 1995

Code	Treatment	Total grain wt.			Wt. of large grains			No. of grains			No. of large grains		
		Irrigation level			Irrigation level			Irrigation level			Irrigation level		
		50%	75%	100%	50%	75%	100%	50%	75%	100%	50%	75%	100%
A	E <sub>1</sub>	11.6	13.3	17.5	7.7	9.0	11.6	61	67	87	32	36	46
B	E <sub>3</sub>	15.5	16.9	19.8	10.0	11.2	13.4	78	84	93	42	47	52
C	E <sub>6</sub>	14.6	14.5	16.6	9.7	9.9	10.8	73	74	83	36	41	42
D	F <sub>1</sub>	14.4	13.2	13.7	9.7	9.3	9.1	77	66	67	39	38	36
E	F <sub>3</sub>	13.5	16.7	17.6	8.8	11.1	11.7	67	80	87	35	46	48
F	F <sub>6</sub>	11.4	15.3	15.9	7.7	9.9	10.7	61	81	78	33	40	44
G	Control	16.2	16.0	17.6	10.7	10.7	12.1	80	77	82	43	41	49
	<i>F</i> (prob.)	0.014	0.408	0.400	0.007	0.520	0.411	0.073	0.355	0.427	0.118	0.081	0.309
	LSD ( <i>P</i> = 0.05)	2.83	—	—	1.68	—	—	—	—	—	—	—	—
	A–C	13.9	14.9	18.0	9.1	10.1	11.9	71	75	88	37	41	47
	D–E	13.1	14.9	15.7	8.7	10.1	10.5	68	76	77	36	41	43
	<i>F</i> (prob.)	0.280	0.980	0.990	0.321	0.962	0.125	0.432	0.965	0.081	0.500	0.890	0.258
	A × D	13.0	13.3	15.6	8.7	9.2	10.4	69	67	77	36	37	41
	B × E	14.5	16.8	18.7	9.4	11.2	12.6	73	82	90	39	47	50
	C × F	13.0	15.3	16.3	8.7	9.9	10.8	67	78	81	35	41	45
	<i>F</i> (prob.)	0.400	0.093	0.155	0.580	0.138	0.122	0.770	0.122	0.191	0.511	0.146	0.111

E<sub>1</sub>, E<sub>3</sub> and E<sub>6</sub>: application of 1, 3 and 6  $\text{kg ha}^{-1}$  glycinebetaine at full bloom (stage R2).

F<sub>1</sub>, F<sub>3</sub> and F<sub>6</sub>: application of 1, 3 and 6  $\text{kg ha}^{-1}$  glycinebetaine at pod initiation (stage R3).

wt., weight.

rainfall. Daytime maximum and minimum relative humidities were 35% at 0900 h and 25% at 1800 h.

In the 50% irrigation regime, the application of

Table 9

Effect of rate of glycinebetaine application on yield ( $\text{g plant}^{-1}$ ) and selected yield components of soybean, cv. Manark at Kununurra, Western Australia, in 1995, expressed as proportion to control (%). Data was pooled for the 75 and 100% irrigation levels at both times of application. Within rows, numbers with same letters are not significantly different at *P* = 0.05 (LSD test)

Parameter	Glycinebetaine rate ( $\text{kg ha}^{-1}$ )		
	1	3	6
Fresh weight of pods	104.3 a	121.1 b	105.8 a
Dry weight of pods	86.8 a	105.7 b	95.6 ab
Weight of total grains	87.3 a	107.6 b	96.7 ab
Weight of large grains	87.3 a	106.3 b	94.8 ab
No. of grains	90.9 a	109.8 b	102.8 ab
No. of large grains	89.4 a	109.6 b	97.1 ab

glycinebetaine reduced the fresh weight, dry weight and number of pods (Table 7), and weights of total and large seeds (Table 8), in contrast to the 75 and 100% irrigation levels, where its application often resulted in marginal improvements. These results indicate that when applied at pod initiation in the 75% watering regime, 3  $\text{kg ha}^{-1}$  glycinebetaine resulted in a grain yield that was 22% greater than when 1  $\text{kg ha}^{-1}$  was applied in the optimum watering regime (Table 8). Further analysis of data detected a significant positive response in the fresh weight of pods when 3  $\text{kg ha}^{-1}$  glycinebetaine was applied in the 75% irrigation regime (Table 7). Analysis of data pooled (as a result of similarity) for the 75 and 100% irrigation levels showed increases in dry weight of pods, weight of total and large seeds, and number of seeds following the application of 3  $\text{kg ha}^{-1}$  glycinebetaine (Table 9).

## 5. Discussion

Results from experiments in Florida showed the deleterious effects of drought in reducing net photosynthesis, nitrogen fixation, leaf expansive growth, biomass accumulation, radiation use efficiency, and increasing leaf resistance of two soybean cultivars that differ in susceptibility to drought.

The results from the soil drying experiment provided important clues to the physiological role of glycinebetaine in possibly improving plant performance under conditions of drying soil. The data revealed that the transpiration rate of glycinebetaine treated plants was decreased in all conditions to 85% of untreated plants, strongly indicating an anti-transpirant effect of glycinebetaine treatment. The slower transpiration rate allows the plant to access water for a longer period and exhibit greater photosynthesis and nitrogen fixation rates as the soil dried. The hypothesized anti-transpirant effect of glycinebetaine could be important under field conditions. The maximum benefit of a glycinebetaine treatment would seemingly be observed under prolonged drought where the benefit of the conserved soil water would be fully expressed. On the other hand, intermittent drought periods might obscure, if not reverse, the benefit of the anti-transpirant effect, with the penalty likely to be slightly decreased photosynthesis rates under well-watered conditions.

The field experiments tend to reflect the mixed response anticipated by an anti-transpirant effect of glycinebetaine. Leaf resistance following the application of 3 kg ha<sup>-1</sup> glycinebetaine was expectedly greater than the control, judging from the greenhouse results. It is not clear why stressed plants treated with glycinebetaine often had higher leaf resistance, and yet maintained higher photosynthesis rates than the control plants. There is an indication that glycinebetaine reduces stomatal aperture, hence the anti-transpirant property. Furthermore, in contrast to our findings, greenhouse data (personal communication, Susanne Somersalo, Department of Plant Production, University of Helsinki), shows that in the course of 22 h, the rate of water loss declined slower in glycinebetaine-treated, water-stressed, transgenic tobacco plants (even though leaf resistance was lower) than in the non-treated control. This suggests a glycinebetaine-mediated amphiphilic site binding

(as proposed by Schobert, 1977) of cell water, thus preventing cell dehydration. The resultant superior cell water status could have affected the efficiency of physiological processes. This aspect of our results lends itself to further studies.

The leaf area benefit at 43 DAS, following the application of 3 kg ha<sup>-1</sup> glycinebetaine in the field, could be associated with the hypothesized anti-transpirant effect of glycinebetaine. The observed trend of enhanced photosynthesis and nitrogen fixation of both water-stressed and seemingly well-watered Biloxi (and to some extent Cook) following glycinebetaine application, could account for the apparent increases seen in leaf area development and dry weight. In a situation of drought-induced stomatal closure, the maintenance of physiological processes which seems to be indicated by glycinebetaine-treated plants, could have improved the performance of the plants.

The similar response of plants in the 75 and 100% water regimes presents soybean cv. Manark as being moderately sensitive to drought. The significant reductions of biomass and grain yield which were associated with drought in the Australian experiments are consistent with the report by Frederick et al. (1991). There was evidence of interaction between glycinebetaine and watering regime, such that 3 kg ha<sup>-1</sup> glycinebetaine  $\times$  a 25% reduction in quantity of water, more than compensated for optimum irrigation  $\times$  1 kg ha<sup>-1</sup> glycinebetaine. Similar results have been reported for maize and sorghum (Agboma et al., 1996a). The negative effects of glycinebetaine on the severely stressed plants could mean that there is a limit of water deficit to which glycinebetaine application could be of positive value. This also seem to vindicate the adoption of foliar application of glycinebetaine where low soil water status could render root absorption ineffective. Rewatering the plants three times during experimentation may have limited the expression of the glycinebetaine treatment. It is expected that yield should increase between none and 1 kg ha<sup>-1</sup>. It seems a threshold concentration of glycinebetaine is required for positive effects. A similar trend was observed in field grown lupins, where application of low rates of glycinebetaine did not improve yield (Agboma et al., 1996c). The seed yield increase following the application of 3 kg ha<sup>-1</sup> glycinebetaine could be associ-

ated with the greater number of seeds filled and more large seeds.

The relatively high concentrations in the controls, suggest that the 2 cultivars accumulate glycinebetaine, even under seemingly well-watered conditions. The amounts are, however, not comparable to those of well-known accumulators (Takhtajan, 1980; Wyn Jones and Storey, 1981), and so could be classified as low-accumulators. The observed high residual amounts in Biloxi after 4 weeks of treatment, which is inconsistent with earlier reports that it is translocated (Ladyman et al., 1980; Hanson and Wyse, 1982; Mäkelä et al., 1996b), might be attributed to de novo synthesis. The incidence of leaf scorching in plants treated with 6 kg ha<sup>-1</sup> glycinebetaine, compared to 3 kg ha<sup>-1</sup> supports earlier reports (Agboma et al., 1996a; Mäkelä et al., 1996a) that high doses of glycinebetaine could create temporary ionic imbalances in the leaves of treated plants.

## 6. Conclusions

Data from greenhouse experiments suggest that glycinebetaine has anti-transpirant properties that could improve physiological performance of plants under sustained soil water deficit. The pattern of response of soybean growth and yield indicates that 3 kg ha<sup>-1</sup> (between 7 and 12 mM solution) glycinebetaine may be an optimum dose, and the response is independent of time of application. The resultant increase in phytomass following glycinebetaine application, could have subsequently affected the amount of assimilate available to the growing pods. Results of this study indicate that exogenous glycinebetaine has the potential to confer drought tolerance properties on soybean and reduce yield losses associated with water stress. Analysis of residual glycinebetaine in leaves portrays soybean as a low-accumulator.

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